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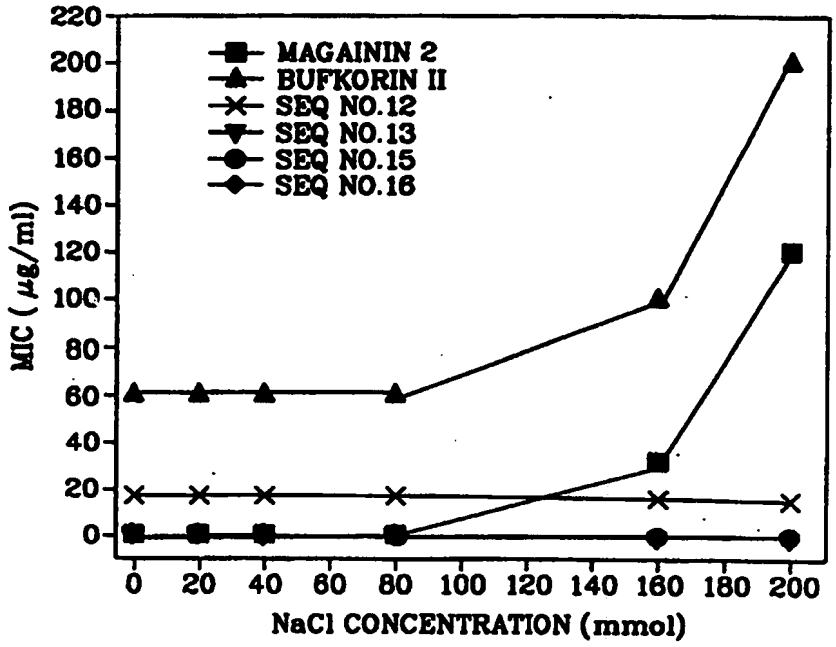
(54) Title: NOVEL PEPTIDES HAVING BIOLOGICAL ACTIVITY



(57) Abstract

The present invention relates to peptides that are more potent than or equally potent as the conventional antimicrobial peptides and has strong antimicrobial activities at high salt concentrations.

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NOVEL PEPTIDES HAVING BIOLOGICAL ACTIVITY

TECHNICAL FIELD

The peptides of the present invention have stronger antimicrobial activities than conventional peptides and have the activity at high salt concentrations.

BACKGROUND ART

The present invention relates to biologically active peptides. Every animal on earth possesses biophylaxis systems to defend or protect itself from the infection by virus or bacteria. One of such systems is a non-specific immunity using antimicrobial peptides.

Antimicrobial peptides are considered as a new type of drug due to the following outstanding properties. Firstly, antimicrobial peptides show stronger antimicrobial activities than conventional antibiotics against a broad spectrum of microorganisms. Secondly, antimicrobial peptides have a high industrial applicability which is beneficial to the human body since the antimicrobial peptides show antimicrobial activity against foreign pathogens without destroying the host cells. Thirdly, there is a smaller chance to develop microbial resistance since the antimicrobial peptides show their activity by a mechanism that is totally different from that of the conventional antibiotics, which have serious problems of developing resistance. Studies on antimicrobial peptides began by isolating cecropin from an insect which has an under-developed immune system. After the first finding, magainin, bombinin

from amphibians, defensins from mammals were isolated. The studies on antimicrobial peptides are actively performed, and to date, about 2,000 antimicrobial peptides have been identified and reported from species ranging from microorganisms to human.

However, there are several barriers to develop the above mentioned antimicrobial peptides as drugs. Firstly, the conventional antimicrobial peptides act at relatively high concentrations. For instance, in case of magainin, an antimicrobial peptide isolated from epidermis of an amphibian, the active concentration is 50-200 μg/ml (Zasloff M. (1987) Proc. Natl. Acad. Sci. USA, 84: 5449-5453) even though it is effective against Gram-positive and Gram-negative bacteria and fungi. This concentration range is quite high considering that the conventional antibiotics act against a specific microorganism in the range 0.1-1 μg/ml. Secondly, the antimicrobial activity of the antimicrobial peptides is sensitive to salt concentration. In case of cystic fibrosis that invades the human lung, for instance, the antimicrobial peptide was not effective due to an abnormal increase of the salt concentrations at the site of invasion (Goldman, M. J. et al. (1997) Cell, 88: 553-560).

Antimicrobial peptides isolated from Korean toad were reported by the present inventors in Biochemical and Biophysical Research Communications 218, 408-413 (1996). These antimicrobial peptides known as buforin I and buforin II showed strong antimicrobial activities against a broad-spectrum of microorganisms including Gram-positive and Gram-negative bacteria and fungi. Buforin I and buforin II also have antimicrobial activities at a concentration of 1-4 μg/ml, which is stronger than that of conventional antimicrobial peptides.

These antimicrobial peptides, however, are also sensitive to salt concentrations. Therefore, it has been desired to develop antimicrobial peptides that have an enhanced antimicrobial activities and are not sensitive to salt concentrations to have antimicrobial activities *in vivo*.

DISCLOSURE OF THE INVENTION

It is an object of the present invention to provide novel biologically active peptides.

Another object of the present invention is to provide peptides that have antimicrobial activities against a wide variety of microorganisms with stronger antimicrobial activities.

It is another object of the present invention to provide peptides that are insensitive to salt concentrations in potentiating the antimicrobial activity.

A further object of the present invention is to provide a secondary structure of peptides that are not sensitive to salt concentration in potentiating the antimicrobial activity.

Another object of the present invention is to provide a precursor peptide that could prepare biologically active peptides.

Still another object of the present invention is to provide cDNA that can code for biologically active peptides.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a secondary structure of buforin II determined by NMR spectrometry in the presence of 50 % trifluoroethanol as a structure-forming agent.

Figure 2 is a graph showing the minimal inhibitory concentration as a function of a salt concentration.

DETAILED DESCRIPTION OF THE INVENTION

The peptide of the present invention comprises a peptide having an amphiphilic α-helix structure.

Also the peptide of the present invention comprises a peptide that has an altered secondary structure of buforin II (Biochemical and Biophysical Research Communications 218, 408-413 (1996)).

The present inventors have shown that the secondary structure of buforin II comprises a random coil (1-4 residue), extended helix (5-10 residue) and normal α-helix (11-21 residue) structures, starting from the N-terminus.

In the structure of buforin II, the peptide sequence having normal α-helix structure (11-21 residue), i.e., PVGRVHRLLRK has a strong antimicrobial activity. The present inventors have identified that a peptide, especially a

peptide with at least the sequence forming the random coil structure (1-4 residue) is removed, has a very strong antimicrobial activity. Therefore, the group of peptides according to the present invention consists of peptides that contain an α-helix structure of buforin II, especially those having the PVGRVHRLLRK sequence. These α-helix forming sequences, for instance the sequence PVGRVHRLLRK, can additionally have amino acids at the C- or N-terminus preferably amino acids forming extended helix or normal helix at the N-terminus or an amidated peptide at the C-terminus.

Another group of peptides according to the present invention comprises a peptide having a repeat unit of $[RLLR]_n$ (n is an integer between 1 and 6), (RLLR being the specific repeat pattern found in the amino acid sequence of buforin II) and preferably peptides where n=2-5.

The peptides can include additional amino acids at the C- or N-terminus, and the amino acid sequence at the N-terminus can include those that do not form a random coil, preferably those forming an extended helix. The group of amino acid sequence, for instance, includes RAGLQFPVG$[RLLR]_1$, RAGLQFPVG$[RLLR]_2$, RAGLQFPVG$[RLLR]_3$, $[RLLR]_3$, $[RLLR]_4$,$[RLLR]_5$, and etc.

The peptides according to the present invention can be synthesized by well-known techniques in the field, for instance, by using an automatic peptide synthesizer or by using a genetic engineering technique. For instance, the peptide can be produced by constructing fusion gene composed of fusion partner and the peptide genes, transforming it into host microorganism,

expressing the fusion protein in the host, cleaving the fusion protein with proteolytic enzyme or chemical agent, and purifying the antimicrobial peptide. For this purpose, for instance, a DNA sequence can be inserted between fusion partner and peptide genes to introduce a sequence encoding processing site which can be cleaved by proteases such as factor Xa and enterokinase, or by chemical agents such as CNBr and hydroxylamine.

To introduce DNA sequence encoding CNBr cleavage site, for instance, fusion partner and antimicrobial peptide genes can be in-frame fused by ligating the fusion partner gene digested at its 3 -end with a restriction enzyme whose recognition sequence contains Met codon (ATG) in their recognition sequence, such as *Afl*II, *Bsm*I, *Bsp*HI, *Bsp*LU11I, *Nco*I, *Nde*I, *Nsi*I, *Ppu*10I, *Sph*I, *Sty*I, or their isoschizomers, and the peptide gene digested at its 5 -end with a restriction enzyme whose cleavage site is compatible with the cleavage site of fusion partner. For another example, to introduce DNA sequence encoding hydroxylamine cleavage site, a DNA sequence encoding Asn-Gly can be introduced between fusion partner and peptide genes. For instance, fusion partner and peptide genes can be in-frame fused by ligating fusion partner gene digested at its 3 -end with a restriction enzyme or its isoschizomer whose recognition sequence contains Asn codon in its recognition sequence, and the peptide gene digested at its 5 -end with a restriction enzyme whose cleavage sequence containing Gly codon can be in-frame fused to the 3 -end of fusion partner by compatible cohesive or blunt end.

The gene structure in the present invention can be introduced into host cell by

cloning it into an expression vector such as plasmid, virus, or other conventional vehicle in which the gene can be inserted or incorporated.

The peptides according to the present invention contain C-terminal amidated forms.

The peptides according to the present invention show strong antimicrobial activities against a wide variety of microorganisms including Gram-negative and Gram-positive bacteria, fungi and protozoa.

The peptides according to the present invention can be administered with other biologically active pharmaceutical preparations such as biologically active chemicals, other peptide, and etc.

The amino acids in the present invention are abbreviated according to the IUPAC_IUB nomenclature as below.

amino acid	abbreviation
Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	E
Cysteine	C
Glutamic acid	D
Glutamine	Q
Glycine	G

Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophane	W
Tyrosine	Y
Valine	V

The invention will be further illustrated by the following examples. It will be apparent to those having conventional knowledge in the field that these examples are given only to explain the present invention more clearly, but the invention is not limited to the examples given.

EXAMPLE 1. Preparation of peptides

According to the sequence given in Table 1, a variety of peptides were synthesized by using an automatic peptide synthesizer and were purified by using a C18 reverse phase high performance liquid chromatography (Waters Associates, USA).

Table 1. Amino acid sequense of buforin II and its derivatives

Peptide	Amino acid sequence
SEQ ID NO. 1	RAGLQFPVGRVHRLLRK
SEQ ID NO. 2	AGLQFPVGRVHRLLRK
SEQ ID NO. 3	GLQFPVGRVHRLLRK
SEQ ID NO. 4	LQFPVGRVHRLLRK
SEQ ID NO. 5	QFPVGRVHRLLRK
SEQ ID NO. 6	FPVGRVHRLLRK
SEQ ID NO. 7	PVGRVHRLLRK
SEQ ID NO. 8	TRSSRAGLQFPVGRVHR
SEQ ID NO. 9	RAGLQFPVGRVHRLLR
SEQ ID NO. 10	RAGLQFPVGRVHRLL
SEQ ID NO. 11	RAGLQFPVGRVHRL
SEQ ID NO. 12	RKGLQKLVGRVHRLLRK
SEQ ID NO. 13	RLLRRLLRRLLRRLLRRLLR
SEQ ID NO. 14	RVHRLLRRVHRLLRRVHRLLR
SEQ ID NO. 15	RAGLQFPVGRLLRRLLRRLLR
SEQ ID NO. 16	RAGLQFPVGRVHRLLRK-NH_2
SEQ ID NO. 17	RAGLQFPVGRLLR
SEQ ID NO. 18	RAGLQFPVGRLLRRLLR
SEQ ID NO. 19	RLLRRLLRRLLR
SEQ ID NO. 20	RLLRRLLRRLLRRLLR

EXAMPLE 2. Estimation of antimicrobial activity

By using the peptides as in Example 1, the minimal inhibitory concentration of the peptides were determined against a variety of microorganisms. Bacteria and fungi were incubated overnight in Miller-Hinton and Saboraud media, respectively, at 37 and 30 °C, respectively, and were inoculated in media for 2 hours to a midlogarithmic phase. After diluting the bacteria and fungi to 10^4-10^5 per 1 ml, they were inoculated into a 96-well plate containing serially diluted peptides and incubated for additional 18 hours. The minimal inhibitory concentration was determined at a concentration that inhibits the growth of the microorganisms by measuring the absorbance. The results are shown in Table 2.

Table 2. Antimicrobial Activity of the peptides

Microorganisms	Minimal Inhibitory Concentrations (μg/ml)											
	Buforin II	Seq No.1	Seq No.2	Seq No.3	Seq No.4	Seq No.5	Seq No.6	Seq No.7	Seq No.8	Seq No.9	Seq No.10	Seq No.11
Gram-positive												
Bacillus subtilis	2	1	4	4	8	18	32	25	>200	12	50	100
Staphylococcus aureus	4	2	8	8	18	62	32	50	>200	50	200	200
Streptococcus mutans	2	1	4	4	8	36	32	25	>200	25	50	100
Streptococcus pneumoniae	4	2	4	4	18	18	32	50	>200	25	100	100
Gram-negative												
Escherichia coli	4	2	2	2	8	36	32	25	>200	12	50	200
Serratia sp.	1	2	2	2	4	18	16	25	>200	12	25	100
Psudomonas putida	4	2	2	2	8	36	32	50	>200	25	50	200
Salmonella typhimurium	2	1	4	4	18	18	64	50	>200	25	50	200
Fungi												
Candida albicans	1	1	8	8	36	62	32	50	>200	50	>200	>200
Cryptococcus neoformans	1	1	8	8	62	62	>100	50	>200	50	100	200
Saccharomyces cerevisiae	1	1	8	8	36	62	>100	50	>200	100	>200	>200

Table 2. - continued

Microorganisms	Minimal Inhibitory Concentrations (μg/ml)										
	Buforin II	Seq No.12	Seq No.13	Seq No.14	Seq No.15	Seq No.16	Marg-ainin 2	Buf(5-13) [RLLR]	Buf(5-13) $[RLLR]_2$	$(RLLR)_3$	$(RLLR)_4$
Gram-positive											
Bacillus subtilis	2	18	2	6	1	1	50	32	4	16	2
Staphylococcus aureus	4	18	1	50	1	1	50	64	8	16	1
Streptococcus mutans	2	36	2	25	0.5	0.5	100	16	16	16	2
Streptococcus pneumoniae	4	18	2	100	1	1	50	32	8	16	2
Gram-negative											
Escherichia coli	4	18	2	100	1	2	100	32	8	32	2
Serratia sp.	1	4	1	3	1	1	25	32	8	16	1
Psudomonas putida	4	36	2	50	1	2	50	32	16	16	2
Salmonella typhimurium	2	18	2	50	1	2	50	16	4	16	2
Fungi											
Candida albicans	1	16	8	>200	2	2	25	32	4	32	8
Cryptococcus neoformans	1	8	8	100	1	1	12	32	4	32	8
Saccharomyces cerevisiae	1	4	8	>200	4	2	25	32	8	32	8

The peptide, RAGLQFPVG$(RLLR)_3$, that has a fused amino acid sequence forming extended <alpha>-helix at the N-terminus of $(RLLR)_3$ showed an especially potent antimicrobial activity, and the peptide $(RLLR)_4$ and $(RLLR)_5$, which has 4 and 5 repetitions of RLLR,respectively, also showed strong antimicrobial activities.

The peptide that had a deletion of the sequence forming a random coil structure from buforin II also showed a potent antimicrobial activity, and the peptide that had an amidation at the N-terminus showed a more potent antimicrobial activity.

EXAMPLE 3. Estimation of antimicrobial activity as a function of salt concentrations

The minimal inhibitory concentration of the peptides was measured as a function of salt concentrations to determine whether the antimicrobial activity is dependent on the salt concentrations. The method of estimating the minimal inhibitory concentration was identical as in Example 2 except that the concentration of NaCl was changed. The result is shown in Figure 2. The antimicrobial activity of the peptides according to the present invention did not vary as a function of salt concentration whereas that of buforin and magainin changed sensitively as a function of salt concentrations.

What is claimed is:

1. Peptides that include the sequence $[RLLR]_n$ (n is an integer between 1 and 6).
2. The peptide according to Claim 1 wherein an extended helix-forming sequence is fused to N-terminus of the peptide.
3. The peptide according to Claim 1 wherein an additional Gly residue is fused to N-terminus of the peptide.
4. The peptides according to Claim 1 wherein the peptides are C-terminal amidated forms.
5. 5. A peptide that contains PVGRVHRLLRK or a peptide that has an equivalent function as the PVGRVHRLLRK sequence and forms an α-helix.
6. The peptide according to Claim 5 wherein the amino acids, that form extended helix or normal helix structure, were fused to N-terminus.
7. The peptide according to Claim 5 wherein the C-terminus of the peptide is amidated.
8. The peptide according to Claim 5 wherein the peptide is comprising one amino acid sequence selected from the group consisting of; RAGLQFPVGRVHRLLRK, RAGLQFPVGRVHRLLRK-amide, RKGLQKLVGRVHRLLRK, RLLRRLLRRLLRRLLRRLLR, RAGLQFPVGRLLRRLLRRLLR, and peptides wherein Gly residue is fused to the N-terminus of said peptides.

FIG. 1

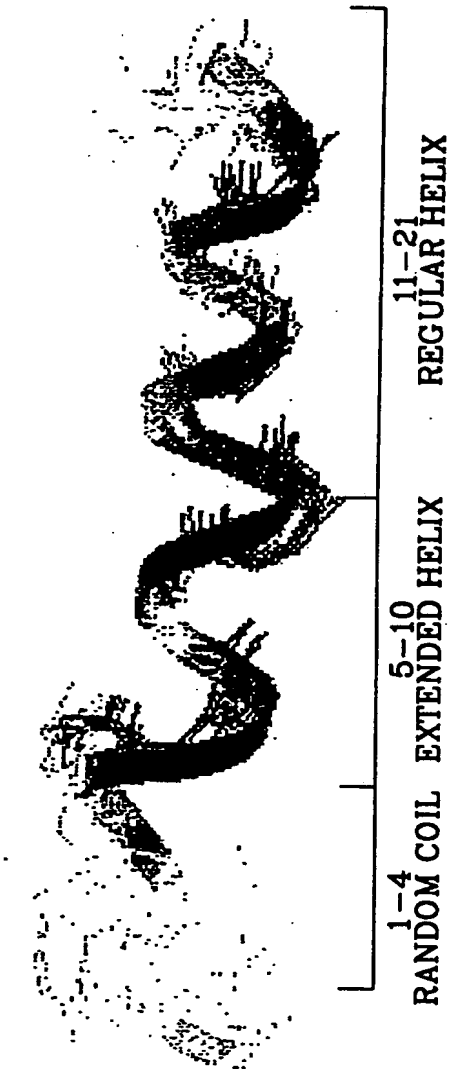
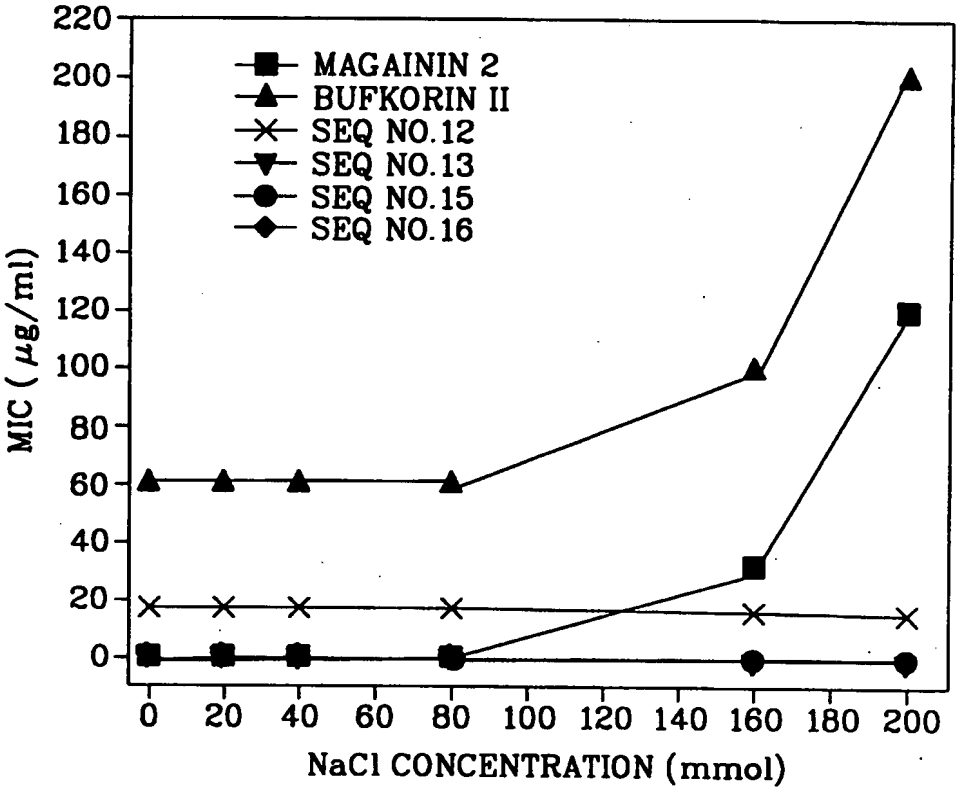


FIG. 2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SEQ NO.13
SEQ NO.15
SEQ NO.16
MIC (μg/ml)
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100
80
60
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20
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NaCl CONCENTRATION (mmol)

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<150> KR 1998-1797
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 99/00036

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: C 07 K 4/00,7/08,14/00; C 12 P 21/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[6]: C 07 K; C 12 P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIL database, Derwent Publications Ltd., London (GB); CAS database, Questel.Orbit.Imagniations, Paris (FR)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	Biochemical and Biophysical Research Communications, Vol.218, 1996, pages 408-413, Article No.0071; Chan Bae Park et al.: "A Novel Antimicrobial Peptide from Bufo Bufo Gargarizans", totality (cited in the application).	1,5 2-4,6-8
X A	Biochemical and Biophysical Research Communications, Vol.229, 1996, pages 381-387, Article No.1814; Hun Sik Kim et al.: "cDNA Cloning and characterization of Buferin I, an Antimicrobial Peptide: A cleavage Product of Histone H2A", totality.	1,5 2-4,6-8
X A	FebsLetters, Vol.398/1, 1996, pages 87-90, Gwan-Su Yi et al.: "Solution Structure of an Antimicrobial Peptide Bufarin II", totality.	1,5 2-4,6-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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